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THE BIOLOGY AND CHEMISTRY OF 'SPHAEROTILUS'

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PROGRESS REPORT

A. Summary Page

(1) WP-00239 The Biology and Chemistry of Sphaerotilus

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(6) Summary statement:

Eleven pure cultures of Sphaerotilus discophorus were isolated from streams, rivers and lakes by appropriate enrichment culture techniques. The morphology of the colonies, filaments and individual cells was investigated in detail. The carbon, nitrogen, mineral and growth factor requirements of S. discophorus were determined. The effects of pH, temperature and oxygen on growth were established. The extent to which iron and manganese are accumulated in the sheaths of S. discophorus was determined by quantitative analyses.

The oxidative patterns of S. discophorus with carbohydrates, amino acids and TCA cycle intermediates were determined and the occurrence of a high degree of oxidative assimilation was established. Large amounts of poly-β-hydroxybutyric acid were found in the cells of both S. discophorus and S. natans. Preliminary investigations were made on the problem of chemoautotrophy in Sphaerotilus.

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B. Detailed Report

The Biology and Chemistry of Sphaerotilus

PART I

STUDIES ON SPHAEROTILUS DISCOPHORUS

I. INTRODUCTION

Although it has been more than a century since filamentous sheathed iron bacteris were first described, our knowledge about this group of organisms is fragmentary and incomplete. Investigators have described the iron bacteria under various names. This problem of taxonomy exists, as Pringsheim (1949b) pointed out, because "cultures, which alone could provide a basis for comparison, are still in great part lacking or insufficient."

Sphaerotilus natans, the most common filamentous sheathed iron bacterium has been studied by many investigators, Kützing, 1833; Cohn, 1875; Molisch, 1910; Linde, 1913; Zikes, 1915; Cataldi, 1939; Lackey and Wattie, 1940; Pringsheim, 1949; Stokes, 1954; Höhnl, 1955; Waitz and Lackey, 1959; Mulder and van Veen, 1962; and by many others.

Our aim was to isolate and study in pure culture another less frequently investigated species, Sphaerotilus discophorus. This organism has been described under many names. Kützing (1843) described it as Leptothrix ochracea, an organism which forms a yellowish red deposit of iron oxide on its sheath. Winogradsky (1888) studied some of these organisms and named them iron bacteria because of their affinity for this metal. According to Pringsheim (1949b), the Leptothrix ochracea of Winogradsky is S. discophorus.

Molisch (1910) isolated pure cultures of a "typical iron bacterium" Leptothrix ochracea using specially prepared peptone and manganese. Schwers (1912) described an organism which makes a thick brown sheath composed of manganese and iron compounds as Megalothrix discophora, and Cholodny (1926) described the same organism under the name of Leptothrix crassa. Later Cataldi (1939) isolated many strains of this organism on solid media and described them under Leptothrix ochracea, L. crassa, L. winogradskii. Pringsheim (1949b) isolated three strains of S. discophorus and made a comparative study of this organism and S. natans. He critically reviewed the literature and proposed a solution to the puzzling nomenclature of this group of bacteria. He recognized S. natans and S. discophorus as being the only two species in the genus Sphaerotilus. According to Pringsheim, Leptothrix, Cladothrix, and Chlamydothrix are different morphological forms of Sphaerotilus. In agreement with Pringsheim (1949a), we shall refer to our cultures as S. discophorus.

There has been virtually no systematic study of S. discophorus. The reason may be the difficulty in isolating this organism in pure culture. In the present investigation 11 pure cultures of Sphaerotilus discophorus were isolated from streams, rivers, and lakes. Their cultural, morphological, nutritional, physiological,

and biochemical properties have been studied. As will be indicated, the results extend considerably our knowledge of S. discophorus.

II. EXPERIMENTAL PROCEDURES AND RESULTS

Enrichment Technique

The hay infusion method of Winogradsky (1888) and Cataldi (1939), with some modifications, was used successfully for the isolation of S. discophorus. The medium for the enrichment cultures contained a mixture of extracted hay, freshly precipitated ferric hydroxide and manganous carbonate. Alfalfa hay was cut into small pieces, thoroughly extracted with large volumes of boiling water several times until the decanted water was almost colorless. The extracted hay was dried at 75 C and stored in closed jars.

A saturated solution of $FeSO_4$ was treated with NaOH to precipitate $Fe(OH)_3$. The precipitate was thoroughly washed until all alkali was removed. Actually, a blue colored $Fe(OH)_2$ precipitate is first formed, which is oxidized readily by air to $Fe(OH)_3$. The precipitate was then mixed with extracted hay, both in excess, and $MnCO_3$ was added. The latter was present in a concentration of about 0.03% in the complete medium. The mixture was placed in glass cylinders and filled with samples of water from rivers, streams, and lakes and incubated at 25 C. It was observed that flasks are not satisfactory for enrichment purposes. Tall cylinders, however, are satisfactory.

In about a week, reddish-brown flakes were seen on the sides of the cylinder near the surface. Microscopically, the reddish-brown material consisted of striking filaments of S. discophorus heavily encrusted with oxides of iron and manganese. The filaments were 10 to 15 μ in width and most of this thickness was due to the inorganic oxide deposit.

Isolation

Characteristic filaments from enrichment cultures were washed with sterile distilled water in a petri dish and then transferred to a white filter paper and washed again with sterile distilled water to remove many of the contaminating organisms. The washed filaments were transferred to another filter paper and the process of washing was repeated. This technique permits the isolation of Sphaerotilus discophorus easily from a mixture of contaminating organisms. The washed filaments were streaked on a medium containing 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.005% $MnSO_4 \cdot H_2O$, 0.05% ferric ammonium citrate, 0.5% Bacto-peptone, and 1.2% agar in tap water. In 2 to 3 days at 25 C small colonies, several millimeters in diameter, were observed on the plates. The colonies had a characteristic dark brown color which permitted easy identification and also differentiation from contaminating bacteria. Usually two main types of colonies were formed on the plates. One type was dark reddish-brown, rough, filamentous, hairlike with curled edges and the other was light brown to yellowish, smooth, watery, without curled edges. No attempts were made to isolate the latter type as it was difficult to be sure whether this was an S or

smooth variant of S. discophorus or a contaminant.

Pure cultures were obtained by restreaking isolated colonies on the peptone-mineral salts agar. Without manganese it was not possible to differentiate colonies of S. discophorus from contaminants. Altogether 11 pure cultures were isolated and identified as S. discophorus by their morphological and cultural properties (Pringsheim, 1949b).

Morphology

Colony

The colonies of S. discophorus are readily recognized by their characteristic filamentous form and reddish-brown to dark brown coloration, due to the deposition of iron and manganese. The hairlike edges of the colonies resembled those of S. natans (Stokes, 1954), although the spreading of the edges are not as extensive. Young colonies are 2 to 5 millimeters in diameter. Occasionally, the hairlike filaments are quite short and the edge of the colony appears almost smooth. These colonial forms are equivalent to the rough or R type, which is common among many bacteria. On nutrient, yeast extract, plate count, and Trypticase soy agar, growth is poor and the cultures tend to dissociate to give rise to colonies which may be colorless, translucent and may have an entirely smooth edge. Such colonies are indistinguishable from those of many other bacteria and represent the smooth or S form of S. discophorus. Pringsheim (1949b) also observed the R to S dissociation in his cultures. In the related species, S. natans, Stokes (1954) observed a similar dissociation. He reported that a low content of organic matter in the medium favors the formation of R types and that the S forms are predominant in richer media. The rough type of colony is made up of sheathed filaments of chains of cells and the smooth type mostly of individual cells. Several factors influenced the R to S dissociation in our cultures. In rich media and in media with a pH of 6.0 to 6.5, cultures dissociated into the colorless smooth type. The latter can be converted back to the dark, rough type by growing the cells in poorer media and in media at pH 7 or slightly above. Also, added thiamin (0.2 µg/ml), was needed in the peptone-mineral salts medium in the presence of glucose for the formation of rough, dark colonies. Without glucose, thiamin was not needed for the formation of the dark colonies. Smoothness was always associated with little or no coloring and roughness with yellow to dark brown coloring.

Filament

The growth of S. discophorus consists of brown filaments composed of rod-shaped cells. The rod-shaped cells, although not visible, lie in chains in the center of the filament encased in a tightly fitting colorless, transparent, and delicate sheath. Iron and manganese oxides are deposited on the sheath during growth and the deposits increase in thickness as the cultures age. The shape of the inorganic deposit is sometimes hemispherical in older cultures and seems to be broken at the juncture of two cells. The filaments of S. discophorus are usually not as long as those of S.

natans. In young cultures the filaments are golden yellow in color and the deposits on the sheaths are continuous. In older cultures the filaments turn reddish-brown to dark brown. Staining is unnecessary for microscopical studies of the filaments. Our observations are in agreement with those of Pringsheim (1949b) in that there is no dichotomous branching of the filaments.

Sheath

The sheath which enclosed the rods is thin, delicate, and transparent. Its refractive index is very close to that of water and it is extremely difficult to detect, therefore, in wet mounts. Cholodny (1926) was not able to observe this primary delicate sheath and he reported that there does not exist a gelatinous sheath composed of organic substances. Pringsheim (1949b) observed the primary delicate sheath which was recognizable where cells of the young filaments were displaced by pressure. The sheath can be seen in preparations negatively stained with nigrosin. Filaments from colonies grown on media without manganese show no manganese oxide deposition on the sheaths. Such colonies and also liquid cultures grown without manganese, but with other minerals, glucose, peptone, biotin, and thiamin, are best for detecting the primary sheath, since manganese oxide obscures the sheath. The primary sheath, as will be shown later, consists primarily of organic material. Its appearance resembles somewhat the sheath of S. natans. The difference between the sheaths of S. natans and S. discophorus lies in the greater width of the former and the ease with which it can be observed.

The secondary covering of the filaments is typically golden brown in color and gets darker and thicker with the age of the culture as previously indicated. This covering which may be called a secondary sheath consists of oxides of iron and manganese and the dark brown color is due primarily to the manganese present. Molisch (1910) obtained well-developed dark brown sheaths in manganese-peptone agar prepared with Prague water, but not with media prepared with Vienna water. Pringsheim (1949b) remarked that this was due to insufficient iron in Vienna water. It was noticed that in our cultures grown without manganese there was only a relatively thin light yellow deposit on the primary sheath due to iron deposition.

The mature oxide encrusted sheaths described by Schwers (1912), Cholodny (1926), Cataldi (1939), and Pringsheim (1949b) are much wider than those of S. natans. Cholodny found the sheath of S. discophorus to be 10 to 15 μ wide, Cataldi 16 μ, and Pringsheim, 23 μ. In older cultures, according to our estimate, they may be as much as 20 μ in width.

Cells

The rod-shaped cells are encased in the tightly fitting primary sheath. In young cultures, chains of cells can be readily seen moving out from the oxide encrusted sheath.

For measurement of the dimensions of the cells, cultures were grown in a medium which contained minerals (without $MnSO_4 \cdot H_2O$),

peptone, glucose, biotin, and thiamin. The concentrations of these particular ingredients are given on the next page. In this medium the growth consists mainly of individual, actively motile rods. These rods are about 0.5 to 0.9 μ wide and 2 to 6 μ long. The individual rods from cultures grown in various media were found to be of the same size. According to Cholodny (1924), the size of this organism is 0.5 μ in diameter and 1 to 2 μ in length. Cataldi (1939) reported the cells to be 0.5 to 1.3 μ in width, and Pringsheim (1949b), 5 to 10 μ or more in length. Our measurements are in fairly good agreement with those of the latter investigators. The cells of S. discophorus are considerably smaller than those of S. natans. The individual cells of S. natans (Stokes, 1959) are from 2.5 to 16 μ long and 1.2 to 1.8 μ wide. S. discophorus is gram-negative and the cells are actively motile by means of a single, polar flagellum. The cells contain refractile, sudanophilic granules which are not as prominent as those of S. natans. The chemical nature of the granules will be discussed later.

Nutrition

Sources of Carbon and Energy

The basal medium contained 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.05% ferric ammonium citrate, 0.05% K_2HPO_4, 0.2 μg/ml thiamin, 0.02 μg/ml biotin, 0.05% Bacto-casamino acids, and distilled water. Manganese ($MnSO_4 \cdot H_2O$) was omitted because its presence makes it difficult to judge the amount of growth due to deposition of manganese oxides on the primary sheath material. There was no need for a highly buffered medium since the growth of S. discophorus even in unbuffered medium does not change the pH to any appreciable extent. Moreover, use of phosphate buffer at a concentration higher than 0.05% K_2HPO_4 was somewhat inhibitory. The pH of the medium was adjusted to 7.1. Casamino acids were used as the nitrogen source because of the inability of S. discophorus to utilize inorganic nitrogen for growth. Casamino acids in a concentration of 0.05% were enough to permit reasonably good growth when the medium contained a utilizable carbon and energy source. Without the added carbon source growth was barely visible. In this way it was possible to judge the utilization of different carbon sources by visual observation.

The basal medium was distributed in 10-ml amounts in 50-ml Erlenmeyer flasks and autoclaved. To each flask was added 1 ml of a separately autoclaved solution of the carbon source to give a final concentration of 0.3% in the medium. Acids were neutralized prior to addition to the basal medium. Ethyl alcohol was sterilized by filtration.

The basal medium without any added carbon source served as the control. For inocula, the cells were grown in the usual medium containing peptone, but no glucose. This eliminated the possibility of carbon source carry over with inoculum. One-tenth of 1 ml of a broth culture was added to 5 ml of sterile distilled water and 1 drop of the resulting suspension was added to each flask of medium. The flasks were incubated at 30 C and growth was judged by visual observation. Growth occurs primarily as a surface pellicle.

Sometimes when growth is poor, it occurs as a thin, granulated film clinging to the sides or bottom of the flask. The main body of the medium always remains clear. Maximal growth usually occurs in 2 to 3 days at 30 C. It is important not to disturb the flask once the surface pellicle is formed, otherwise growth is retarded. Incubation after maximal growth has taken place is always followed by rapid lysis of the cells.

Eight strains were employed in these studies. It was observed that S. discophorus can utilize a variety of organic compounds as sources of carbon and energy (Table 1). All of the strains utilized glucose, fructose, galactose, mannitol, maltose, and glycerol; five strains utilized sorbitol, sucrose, and ethanol; four strains, lactate; and one strain, succinate. There was very slight growth of some strains with arabinose, xylose, erythritol, and acetate and no growth with lactose, formate, propionate, butyrate, citrate, and benzoate. Of all the carbon sources tested, glycerol appeared to support the most growth.

Good growth was obtained also in a medium containing 0.5% Bacto-peptone, 0.2% glucose, 0.05% ferric ammonium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.2 µg/ml thiamine, 0.02 µg/ml biotin, and distilled water (pH 7.1). The addition of $MnSO_4$ was not necessary for growth in this medium although it was essential for the development of the typical dark brown color. Omission of glucose or vitamins markedly reduces growth. The medium yields about 1 gram (dry weight) of S. discophorus per liter in 2 days at 30 C. When the concentration of glucose was increased to 1% or more, growth was almost completely inhibited. This inhibitory effect of glucose at high concentration could not be eliminated by use of filter-sterilized glucose. Little or no growth occurs in relatively rich media such as nutrient, yeast extract, Trypticase soy, or brain heart infusion broth or on the corresponding agar media.

Our data indicate therefore that S. discophorus can utilize a number of organic compounds as sources of carbon and energy, and in this respect it is similar to S. natans (Stokes, 1954). Both organisms can utilize glucose, galactose, mannitol, sorbitol, sucrose, maltose, glycerol, and ethanol as sources of carbon and energy. They are also comparable with respect to their inability to utilize lactose, xylose, arabinose, benzoate, and propionate for growth.

Sources of Nitrogen

The basal medium was the same as that used in the carbon and energy experiments except that the peptone was omitted and 0.005% $MnSO_4 \cdot H_2O$ was added. Also, glucose (0.3%) was added as the carbon and energy source. The inorganic nitrogenous compounds were used in a concentration of 0.1%. The experiments were performed in the same manner as the carbon experiments. Two series of control were used. One series contained the basal medium, glucose, and no nitrogen source, and the other series contained the basal medium, glucose, and an organic nitrogen source, either Bacto-peptone or casamino acids in a concentration of 0.3 to 0.5%.

TABLE 1.--The utilization of various carbon compounds by Sphaerotilus discophorus

Substrate[a]	Strains							
	16	21	32	35	36	39	42	43
Control[b]	-[c]	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Sorbitol	+	+	±	+	+	+	+	-
Sucrose	±	±	+	+	+	+	+	-
Maltose	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-
Arabinose	±	±	±	±	-	-	-	±
Xylose	±	±	±	±	-	-	±	-
Erythritol	±	±	±	±	-	-	±	-
Glycerol	+	+	+	+	+	+	+	+
Ethanol	+	+	+	+	±	+	±	±
Formate, Na	-	-	-	-	-	-	-	-
Acetate, Na	±	-	-	-	-	-	±	-
Propionate, Na	-	-	-	-	-	-	-	-
Butyrate, Na	-	-	-	-	-	-	-	-
Lactate, Na	+	±	±	+	+	+	-	-
Succinate, Na	-	-	-	+	-	-	-	-
Citrate, Na	-	-	-	-	-	-	-	-
Benzoate, Na	-	-	-	-	-	-	-	-

[a]All substrates except ethanol separately sterilized; 0.3% final concentration; ethanol filter sterilized.

[b]Basal medium without carbon source.

[c]- = no growth; ± = very slight growth; + = good growth.

Repeated attempts to obtain growth with inorganic nitrogen such as nitrate or ammonium salts were always unsuccessful. Our results are in contrast to those of Cataldi (1939) who obtained good growth of Leptothrix with ammonium nitrogen. In the related species, S. natans, Stokes (1954) reported that inorganic nitrogen in the form of ammonium or nitrate nitrogen did not permit growth in most cases, although he obtained growth in few instances when sucrose, glycerol, or succinate was the carbon source.

Since S. discophorus utilizes casamino acids (prepared by either acid or enzyme hydrolysis), as nitrogen source, attempts were made to grow the strains in a mixture of 20 amino acids. The total amino acid concentration was 0.4%, and the individual amino acids added were in the concentration of 20 mg per 100 ml. The procedure followed was that of Stokes et al. (1945). It was rather strange that all our attempts to obtain growth with mixtures of amino acids ended in failure and this aspect needs further investigation.

Mineral Requirements

Iron was found to be essential and manganese nonessential for the growth of S. discophorus. Manganese was added as a means of identifying S. discophorus by the development of the typical dark brown color. The role of iron and manganese will be discussed in detail later in the section on physiology. Omission of either $MgSO_4$ or $CaCl_2$ or both from standard peptone medium reduced growth somewhat and their effect was more pronounced in media containing only 0.1% peptone.

Growth Factor Requirements

The methodology used in these studies was the same as that used for the carbon and nitrogen source determinations. The medium contained the usual minerals, glucose, and an acid hydrolysate vitamin-free casein in place of peptone. The control flasks contained peptone instead of casein hydrolysate. The vitamins used, in micrograms per ml of medium, were thiamin, riboflavin, nicotinic acid, pantothenic acid, 0.2; pyridoxin, 1.0; para-aminobenzoic acid, 0.4; pteroyl glutamic acid, 0.01; and, biotin, 0.02. A series of flasks were prepared in which each vitamin was omitted in turn from the mixture of all of them. The inocula for these studies were taken from 2-day-old agar slant cultures. The danger of carry over of growth factors, other than biotin and thiamin, with the inoculum was reduced by using cells grown on the minerals, vitamin-free casein hydrolysate, thiamin, and biotin medium. It was found that all of the strains of S. discophorus require thiamin and biotin for growth (Table 2). In the absence of thiamin, no growth was obtained. Only slight growth occurred in the absence of biotin. The need for biotin was rechecked by using washed inocula. It was observed that after 6 days of incubation only two strains out of six showed very slight growth without biotin; the remaining four strains did not grow. In the presence of 0.001 µg/ml of biotin, however, good growth occurred with all strains within 2 days at 30 C. These results indicate that biotin is also required by S. discophorus for growth.

TABLE 2.--Growth factor requirements of *Sphaerotilus discophorus*

Media	Strain			
	32	36	39	43
Basal[a]	4+[b]	4+	4+	4+
Basal minus thiamin	-	-	-	-
Basal minus riboflavin	4+	4+	4+	4+
Basal minus nicotinic acid	4+	4+	4+	4+
Basal minus pantothenic acid	4+	4+	4+	4+
Basal minus pyridoxine	4+	4+	4+	4+
Basal minus para-amino benzoic acid	4+	4+	4+	4+
Basal minus pteroylglutamic acid	4+	4+	4+	4+
Basal minus biotin	1+	1+	±	±

[a]Contains the usual minerals, 0.5% vitamin-free casamino acids, 0.01% tryptophane, 0.0025% cysteine, 0.3% glucose, and all of the vitamins mentioned in the table.

[b]- = no growth; ± = very slight growth; 1+ to 4+ = increasing amount of growth. Results recorded after 3 days of incubation.

Pringsheim (1949b) reported that soil extract stimulated the growth of S. discophorus as well as S. natans in dilute beef extract and other media. It may be this stimulation of S. discophorus is due to thiamin and biotin present in the soil extract. The situation is different, however, for S. natans since a mixture of $MgSO_4$, $CaCl_2$, and $FeCl_3$ could replace soil extract (Stokes, 1954). In this respect, the two species are strikingly different.

Maintenance of Stock Cultures

The stock cultures were kept on a medium containing 0.5% peptone, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot H_2O$, 0.005% $FeCl_3 \cdot 6H_2O$, 0.05% ferric ammonium citrate, 1.2% agar, and tap water. Glucose was not added to the medium since it tends to stimulate dissociation of the cultures into rough and smooth types. Dissociation makes it difficult to ascertain the purity of the stock cultures. Also, biotin and thiamin were not added as these are supplied by the peptone. The stock cultures were maintained at 10 C, and transferred at least once a month. Cultures older than a month grow poorly on subculture and prolonged incubation of about 7 days is necessary. Stock cultures stored at 0 C are difficult to revive after 1 month. Cold somehow injures the cells.

Physiology

Role of Iron

Winogradsky (1888) emphasized the importance of iron for this group of organisms. He concluded that the iron bacteria utilize for their metabolism the energy derived from the oxidation of ferrous iron to the ferric state. Molisch (1910) and Lieske (1911) isolated pure culture of Leptothrix ochracea from natural iron sources on organic media. Molisch also noticed that the presence or absence of manganese was not important for their development. Since their strains could grow on organic media, Molisch and Lieske concluded, in contradiction to Winogradsky, that the organism is a heterotroph. Likewise, Cataldi (1939) grew Leptothrix species on a medium composed of ferric ammonium citrate, glucose, and agar. Also, Pringsheim (1949b) grew S. discophorus in a medium containing beef extract, manganous sulphate, and ferrous ammonium citrate.

Likewise, we also have been able to grow all of our strains of S. discophorus on organic media. Small amounts of iron are indispensable for growth but appear to function catalytically rather than as a source of energy. An increase of $FeCl_3$ from 0.001% to 0.1% did not increase the amount of growth. The further addition of 0.05% ferric ammonium citrate to the basal medium doubled the amount of growth. The specific function of this compound in growth is not known. The chelating properties of the citrate ion may be involved in the growth stimulation.

Many attempts were made to grow S. discophorus autotrophically using various types of iron such as pure iron metal, iron wire, and iron sulphide as the principal energy source in media containing the usual minerals, biotin, thiamin, and nitrogen sources such as ammonium or nitrate nitrogen, or organic nitrogen. No growth was

obtained in any of our experiments even after 6 weeks of incubation at 30 C. Also, some unsuccessful experiments were carried out in closed jars where an atmosphere of CO_2, approximately 10%, and O_2 was maintained. It seems unlikely that S. discophorus is an autotroph because of its ability to grow in organic media, its need for organic nitrogen and vitamins, and its inability to develop in inorganic media containing iron.

Role of Manganese

As previously mentioned, Molisch (1910) noted that manganese was not essential for growth of Leptothrix. Cataldi (1939) obtained better growth of Leptothrix in peptone-manganese acetate medium than in media without the manganese acetate. Pringsheim (1949b) noted that it was advantageous to add 0.002 to 0.005% manganous sulphate to culture media for S. discophorus solely to promote development of the easily recognizable dark colonies.

We also found that $MnSO_4$ was not needed for the growth of S. discophorus although its presence was always essential for the development of the striking brown color. A concentration of 0.005 to 0.01% of $MnSO_4 \cdot H_2O$ was satisfactory. When the manganese concentration was raised above 0.01%, growth was inhibited. There was no growth on agar media with 0.1% manganese. The oxidation of $MnSO_4$ by dense cell suspensions of S. discophorus was studied by manometric techniques and the results were negative. Concentrations of $MnSO_4 \cdot H_2O$ from 5 to 50 µmoles per vessel were tested.

Attempts were made to identify the manganese oxides deposited on the sheath of Sphaerotilus discophorus by X-ray diffraction patterns. The cells were grown in stationary cultures and the thick pellicle which formed was washed with distilled water several times. The washed material was then dried at room temperature in a desiccator under vacuum. Scrapings from dried material were examined with a petrographic microscope between cross polaroids. The material showed anisotropism, indicating that the oxides were present in a definite crystal form. X-ray diffraction also showed a definite pattern, but it was not possible to identify the compounds.

Effect of Temperature on Growth

The medium contained the usual minerals, vitamins, 0.3% glucose, and 0.4% peptone. Fifty-milliliter flasks containing 10 ml of medium were used. All of the strains grew in the range of 10 to 35 C, and the optimal temperature was 25 to 30 C (Table 3). Growth also occurred at 5 C but only after prolonged incubation of about a month. These results are in agreement with those of Cataldi (1939), who reported the optimal temperature between 25 to 28 C, with the exception that Leptothrix winogradskii had an optimum at 37 C.

TABLE 3.--Effect of temperature on the growth of Sphaerotilus discophorus

Temperature, C	Strain											
	32			35			36			39		
	2D[a]	4D	15D	2D	4D	15D	2D	4D	15D	2D	4D	15D
0	-[b]	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
10	-	±	4+	±	1+	4+	-	±	4+	-	1+	4+
15	1+	3+	4+	1+	3+	4+	1+	2+	4+	1+	2+	4+
20	2+	4+		2+	4+		2+	4+		2+	4+	
25	3+	4+		3+	4+		3+	4+		3+	4+	
30	3+	4+		3+	4+		3+	4+		3+	4+	
35	3+	3+		3+	3+		3+	3+		3+	3+	
40	-	-	-	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-	-	-	-

[a] 2D indicates that the observation was made on the second day.

[b] - = no growth; ± = very slight growth; 1+ to 4+ = increasing amount of growth.

Effect of pH on Growth

For the determination of the effect of pH on growth, the same medium as used for the temperature experiments was employed. The pH of the medium was adjusted to various values in the range of 4 to 9.8. McIlvaine's standard buffer solution of one-tenth strength was used for pH 4.0 through 7.9. For pH 8.2, 8.6, 9.0, and 9.8, 0.01 M NH_4OH and HCl buffer was used. Highly buffered media were not necessary since S. discophorus produces very little acid in this medium. The data obtained are presented in Table 4. No growth appeared at pH 4.0, 5.0, 9.0, and 9.8. Slow and little growth occurred at pH 76.0, 6.4, 8.2, and 8.6. Rapid and good growth occurred in the range of pH 7.0 to 7.9. The growth at pH 6 to 7 was colorless and largely of the smooth type even though $MnSO_4$ was present in the medium. In summary, the pH range for growth of all strains is pH 6.0 to 8.6 with an optimum at about pH 7.0 to 8.0.

In another series of experiments, the usual medium without buffer was employed. The desired pH values were obtained by the addition of HCl or KOH. The optimal pH and the pH range for growth in these experiments were the same as in the buffered medium. The growth, however, was somewhat greater.

Cataldi (1949) reported the pH optimum for her strains to be between pH 8 and 8.5. Pringsheim (1949b) maintained his cultures in media at pH 7 to 7.5. In general, there is a good agreement that the pH optimum for this organism is slightly above neutrality.

Effect of Oxygen on Growth

S. discophorus is strictly aerobic and many attempts to grow it anaerobically were unsuccessful. In shaken cultures the growth is granular and dispersed. In stationary cultures the growth occurs primarily as a surface pellicle. Both types of cultivation gave about the same amount of growth.

Presence of Hydrolytic Enzymes and Catalase

For starch hydrolysis the usual medium containing peptone, minerals, and 0.2% soluble starch was used. Agar plates were streaked with a heavy inoculum of S. discophorus and incubated at 30 C. Tests for starch hydrolysis were made on the third day by flooding the surface of petri dish cultures with a saturated solution of iodine in 50% alcohol (Conn et al., 1957). For a positive control, the agar plates were also inoculated with a starch hydrolyzing strain of Bacillus cereus. None of the 11 strains of S. discophorus hydrolyzed starch.

To test for dextrin hydrolysis, the peptone-mineral salts medium plus 0.3% dextrin was used. The plate cultures were flooded with the iodine solution after 3 days of incubation. There were no clear zones outside the areas of growth and the entire plate turned reddish-brown which indicated that the dextrin was not hydrolyzed. All 11 strains were negative.

TABLE 4.--Effect of pH on the growth of Sphaerotilus discophorus

pH	Strain									
	32		35		36		39		41	
	2D[a]	4D	2D	4D	2D	4D	2D	4D	2D	4D
4.0	-[b]	--	-	-	-	-	-	-	-	-
5.0	-	-	-	-	-	-	-	-	-	-
6.0	1+	2+	2+	2+	-	2+	2+	2+	1+	2+
6.4	1+	2+	2+	2+	-	2+	1+	2+	1+	2+
7.0	3+	4+	3+	4+	3+	4+	3+	4+	3+	4+
7.9	3+	4+	3+	4+	3+	4+	3+	4+	3+	4+
8.2	2+	2+	2+	4+	2+	4+	2+	4+	2+	4+
8.6	-	1+	-	2+	-	2+	-	2+	-	1+
9.0	-	-	-	-	-	-	-	-	-	-
9.8	-	-	-	-	-	-	-	-	-	-

[a] 2D indicates that the observation was made on the second day.

[b] - = no growth; ± = very slight growth; 1+ to 4+ = increasing amount of growth.

The absence of cellulose digesting enzymes was demonstrated by placing a piece of sterile moist filter paper on the solidified agar medium of usual composition in petri plates, and streaking the filter paper surface with a heavy inoculum, and incubating for a month at 30 C. Also, filter paper strips immersed in liquid medium were used. In all cases the results were negative for cellulose digestion.

Gelatin liquefaction by S. discophorus was determined by following the standard method prescribed in the "Manual of Microbiological Methods." The results were negative for the 11 strains.

The presence of catalase was tested for by the addition of 3% hydrogen peroxide to cultures of S. discophorus and noting the evolution of oxygen under the low power of the microscope. All the strains were catalase positive.

Intracellular Deposition of Poly-β-hydroxybutyric Acid

Although S. discophorus cells do not contain as many prominent sudanophilic granules as S. natans, the cells are almost entirely filled with some irregularly shaped granules of a diffuse nature. An attempt was made to determine whether these granules are composed of the β-hydroxybutyric acid polymer.

The general method employed for the isolation and identification of the polymer was essentially that reported by Rouf and Stokes (1962) for S. natans. Strains 32, 35, and 42 were employed in these studies. The cells were grown in stationary cultures in media containing the usual minerals, biotin, thiamin, 0.4% glucose, and 0.3% peptone. They were harvested for analysis after about 60 hours of growth at 30 C. It was found that S. discophorus, like S. natans, also accumulates a considerable amount of poly-β-hydroxybutyric acid in the cells. The polymer may account for as much as 40% of the dry weight of S. discophorus which is among the highest yields so far reported for bacteria. The amounts of poly-β-hydroxybutyric acid isolated ranged from 30% of the dry weight of the cells of strain 35, to 38% for strain 32, and 40% for strain 42.

Estimation of the Amount of Iron and Manganese in the Cells and Sheaths of Sphaerotilus discophorus

An attempt was made to determine the amounts of iron and manganese deposited on the primary sheaths of S. discophorus.

Strains 21 and 36 of S. discophorus and 4 of S. natans for comparative purposes were employed in these studies. The medium contained the usual mineral salts with and without $MnSO_4$, and vitamins, 0.4% peptone, and 0.3% glucose. The medium was distributed in 200-ml volume into 2-liter Erlenmeyer flasks. The cultures were grown for 60 hours at 30 C, harvested by centrifugation and washed twice with distilled water. The washed cellular material was then collected in dried and weighed crucibles for dry weight determinations. These were made after at least 18 hours of drying at 90 to 94 C; 400 ml of culture was used for one analysis.

The manganese concentration was determined by the method described in the "Official Methods of the Association of Official Agricultural Chemists" (1955). The procedure for the iron analysis was taken from Diehl and Smith (1952). Table 5 shows the results of these analyses. The values as percentage of ash content are included because ashing was a necessary part of the procedure for the assay of both metals.

Iron accounts for 4.25 to 7.10% and manganese 1.12 to 1.43% of the dry weight of S. discophorus when the organism is grown in the presence of both of these metals. Thus, iron is much more abundant than manganese. This is somewhat surprising since the filaments are dark brown which is the color of manganese oxides rather than light yellow, the color of iron oxides. The possibility that much of the iron is within the cells rather than on the sheaths cannot be excluded but seems unlikely. E. coli for example contains only 0.226% iron (Guillemin and Larson, 1922) and Bacillus coherens only 0.004% manganese (Curran, Burnstetter, and Meyer, 1943) on a dry weight basis.

Since the medium contained only 1.26% of iron and 0.38% manganese, the much higher values found in S. discophorus indicate that the organism concentrates these metals on the sheaths. Omission of $MnSO_4$ from the medium results in the formation of filaments which do not contain manganese.

S. natans differs from S. discophorus in that the filaments do not accumulate manganese even when grown with $MnSO_4$. Also, S. natans accumulates less than 1% iron compared to 4 to 7% for S. discophorus.

Since S. discophorus accumulates rather large amounts of iron, the term "iron bacterium" which has been applied to it seems appropriate. Whether the iron can serve as an energy source for growth, however, is questionable.

Oxidative Metabolism

The oxidative metabolism of Sphaerotilus discophorus has not been previously investigated. In the related species, S. natans, as much as 70 to 80% of carbohydrates are oxidatively assimilated and this process is resistant to inhibition by 2,4-dinitrophenol (Stokes, 1954). L-glutamic acid, L-aspartic acid, L-leucine, DL-alanine, and L-proline were also oxidized.

In our studies, conventional manometric techniques were employed. Unless otherwise indicated, the cells were grown in a medium containing 0.3% glucose, 0.4% peptone, vitamins, and the usual mineral salts except $MnSO_4$. In most of the experiments, strains 32 and 35 were used, although strains 36, 39, and 42 were also used in some experiments. The cells were grown at 30 C on a shaker for 15 to 48 hours. They were harvested by centrifugation, washed twice with 0.02 M phosphate buffer, pH 7.1 and resuspended in the same buffer to a Klett density of 380 units. Unwashed and singly washed cells were also used in some experiments.

TABLE 5.--Iron and manganese content of Sphaerotilus

Organiam[a]	Medium[b]	Iron		Manganese	
		% of dry wt	% of ash	% of dry wt	% of ash
S. discophorus, strain 21	with Mn	5.68	35.7	1.43	8.99
	without Mn	4.25	36.0	0.0	0.0
S. discophorus, strain 36	with Mn	5.44	38.4	1.12	7.96
	without Mn	7.10	59.1	0.0	0.0
S. natans, strain 4	with Mn	0.71	17.6	0.01	0.30
Uninoculated medium	with Mn	1.26	12.8	0.38	3.73

[a]The cells were grown in stationary cultures for 60 hours at 30 C.

[b]The medium contained 0.4% Bacto-peptone, 0.3% glucose, biotin, thiamin, and the usual minerals except that the 0.005% $MnSC_4 \cdot H_2O$ was omitted in some instances as indicated.

Initially, lysis of the cell suspensions in 0.1 M phosphate buffer was encountered. These preparations had no activity. After considerable experimentation, it was found that the addition of separately sterilized glucose or other carbon source to the growth medium and the use of lesser concentrations of phosphate in the suspending buffer, i.e., 0.02 M phosphate, prevented rapid lysis.

Stokes (1954) reported that S. natans has a high endogenous respiration rate and to avoid this he aerated the harvested cells for 3 to 5 hours by bubbling a slow stream of air through the cell suspensions. The endogenous respiration rate of S. discophorus also was high but the aeration of cells in phosphate buffer for 2 to 3 hours tended to destroy the oxidizing capacity of the cells.

Four to 8 µM of substrate contained in 0.1 or 0.2 ml of neutralized solution was placed in the side arm of the Warburg vessel. In the center well was placed 0.2 ml of 10% KOH and a folded strip of filter paper to absorb CO_2. To the main compartment, 2 ml of cell suspension (380 Klett units) was added. The gas phase was air and the temperature of the bath 30 C.

The oxidation of carbohydrates and related compounds, amino acids, and fatty acids was investigated. Data on the rate and extent of oxidation of some of these compounds are given in Table 6.

Of the 20 amino acids studies, only glutamic and aspartic acids, serine, alanine, and glycine were slightly oxidized. Among the carbohydrates and related compounds, S. discophorus oxidized those compounds which were utilized as sources of carbon and energy. In all cases only a small amount of the substrate usually less than 10% was oxidized. Although sucrose is oxidized more rapidly than glucose, the latter, in turn, is oxidized more rapidly when the cells are initially grown in a glucose medium.

In the case of glucose, tests were made for residual sugar at the end of the manometric experiments by the anthrone method (Umbreit, Burris, and Stauffer, 1957). It was found that 90 to 93% of the glucose initially present had disappeared, while only 5 to 9% of the glucose was oxidized as indicated by O_2 consumption. It would appear, therefore, that most of the substrate is oxidatively assimilated. The addition of 5×10^{-4}, 5×10^{-5}, 5×10^{-6} M 2,4-dinitrophenol or sodium azide did not increase the extent of oxidation. At the higher concentration of both inhibitors, however, oxidation was inhibited below the endogenous level.

Sphaerotilus discophorus resembles Rhizobium meliloti in its high assimilatory powers (Burris and Wilson, 1942). It was found that when all of the glucose had disappeared, R. meliloti 131 had taken up oxygen equivalent to only 8.6% of the amount necessary for the complete oxidation of the glucose supplied. The addition of M/10,000 2,4-dinitrophenol increased the oxidation to only 13.4%.

The results of the experiments with members of the tricarboxylic acid cycle are also presented in Table 6. These are also oxidized except for citrate.

TABLE 6.--The rate and extent of oxidation of various compounds by Sphaerotilus discophorus

Substrate	O_2 Consumed µl/hr/vessel[a]	% Oxidation
8 µmoles		
Glucose	56	11
Fructose	60	12
Galactose	30	5
Mannitol	23	4
Sucrose	83	8
Maltose	29	5
Lactose	0	0
Arabinose	0	0
Xylose	0	0
Glycerol	18	4
Butyrate, Na	0	0
Citrate, Na	0	0
α-ketoglutarate, Na	12	4
Succinate, Na	40	11
Malate, Na	6	2
Oxaloacetate, Na	7	3
Pyruvate, Na	30	8
Acetate, Na	20	8
4 µmoles		
Glycine	5	1
Alanine	10	4
Serine	28	8
Aspartate, Na	6	2
Glutamate, Na	30	12

[a]Endogenous oxidation subtracted.

The extent of oxidation of various substrates remained the same even when the substrate concentration was increased to 100 or 300 µM. At these higher substrate levels the oxidation of acetate, pyruvate, and succinate was inhibited.

The oxidation of sucrose in the range of pH 4 to 9.2 was investigated. For pH 4 and 5, McIlvaine's standard buffer of one-tenth strength was used and for pH 6, 7, and 8, 0.02 M phosphate buffer and for pH 9.2, 0.02 M tris buffer. These are the initial pH values of the buffers in the vessels. Each vessel received 8 µM of sucrose. It was found that the oxidation of sucrose was maximal at pH 7 and decreased at both pH 6 and 8. There was no oxidation of substrate at pH 4, 5, and 9.2. The effect of pH on oxidation is very similar to its effect on growth and the optimal pH for oxidation as well as for growth lies between pH 7 and 8.

III. DISCUSSION

The ease and the regularity with which S. discophorus was isolated from natural habitats suggests that the organism is widely distributed in nature. The same is true also for the related species, S. natans (Pringsheim, 1949b; Stokes, 1954; Höhnl, 1955). It can be concluded that members of the genus Sphaerotilus are of common occurrence. Sufficient information has now accumulated on satisfactory methods for the isolation of Sphaerotilus to permit systematic surveys of the distribution of these organisms in nature. Such surveys, which have not as yet been made, are necessary for the firm establishment of the role of Sphaerotilus in the transformation of matter in nature.

S. discophorus is similar to S. natans in many of its properties and also strikingly different in several aspects. Both species grow on solid media as distinctive filamentous colonies which can dissociate into an S or smooth form. Both consist of rod-shaped cells enclosed in tightly fitting, transparent, organic sheaths or envelopes. The cells of both species are motile by means of a single, long, polar flagellum and also accumulate large amounts of poly-β-hydroxybutyric acid internally as sudanophilic granules. Both species tend to accumulate morphologically distinct deposits of iron oxide on the primary sheath. Both species grow well in dilute organic media and metabolize a similar variety of carbohydrates, fatty acids, amino acids, and related compounds.

The two species, however, differ in a number of properties. The colonies of S. discophorus become dark brown on manganese containing media owing to the deposition of manganese oxides, whereas those of S. natans do not and, therefore, remain colorless. The filaments of S. discophorus are much thinner than those of S. natans and the individual cells are also thinner and shorter. The primary sheath of S. discophorus is more difficult to see under the microscope than that of S. natans. This suggests that there may be differences in their chemical composition. Correlated with the darkening of S. discophorus colonies on manganese containing media is the deposition of manganese oxides in a thick, morphologically distinct layer on the primary sheath of this species. This phenomemon does not occur in S. natans.

Also, the vitamin requirements of S. discophorus for growth are markedly different than those of S. natans. Linde (1913), Cataldi (1939), Lackey and Wattie (1940), and Stokes (1954) were able to grow S. natans in chemically defined media without the addition of vitamins or other growth factors. Recently, however, Mulder and van Veen (1962) reported that cyanocobalamin or vitamin B_{12} stimulates growth of S. natans. In contrast, S. discophorus requires an exogenous source of thiamin and biotin for growth.

The inhibitory effect of glucose at a concentration of 1% or more is difficult to explain. Moreover little or no growth occurs in relatively rich media such as nutrient, yeast extract, Trypticase soy, or brain heart infusion broth or on corresponding agar media. Wuhrmann (1949) found that the inhibition of Sphaerotilus by undiluted sewage, raw or sterile, was due to the presence of high concentrations of tryptophane, cystine, and methionine. It was reported that each of these amino acids was present in a concentration of 500 µg per liter and that a 20-fold dilution of raw sewage was necessary for good growth. High concentrations of these amino acids in ordinary culture may be responsible, therefore, for the observed growth inhibition. However, Stokes (1954) obtained good growth of S. natans with tryptophane and cystine at a concentration of 1,000 mg per liter.

The inability of S. discophorus to utilize ammonium or nitrate nitrogen for growth is interesting. Cataldi (1939) reported that Leptothrix grew well with $(NH_4)_2SO_4$. Lackey and Wattie (1940) also obtained good growth of S. natans with ammonium or nitrate salts but no growth with nitrite salts. Stokes (1954) obtained good growth of S. natans with inorganic nitrogen when glycerol, succinate, or sucrose was the carbon source. With the other carbon sources, such as glucose, galactose, maltose, mannitol, sorbitol, fumarate, butyrate, butanol, sodium lactate, sodium pyruvate, sodium acetate, ethanol, and butanol he obtained no growth with ammonium or nitrate nitrogen. Höhnl (1955) found that alanine and asparagine could support growth of S. natans when an additional nitrogen source such as nitrate was present in the medium. On the whole, the nitrogen requirements of the genus Sphaerotilus need further investigation.

Despite Winogradsky's original contention that Leptothrix (S. discophorus) is a chemoautotrophic organism and can utilize the energy derived from the oxidation of iron for synthesis of cellular material, unequivocal proof is still lacking. As previously indicated, Molisch (1910) and Cataldi (1939) considered this organism to be a heterotroph because it grew only with organic matter. In contrast, Cholodny (1926) did not completely rule out the possibility of chemoautotrophy. Also, Pringsheim (1949b) emphasized that only a small amount of organic matter is sufficient for the growth of Sphaerotilus and that oxidizable inorganic iron and manganese compounds markedly increased growth.

Our nutritional studies show that S. discophorus requires vitamins and organic nitrogen for growth. This speaks against chemoautotrophy. Yet, the striking oxidation and accumulation of

manganese and iron by S. discophorus strongly suggest an important metabolic role for these inorganic oxidations. Moreover, the large stimulatory growth effect of ferric ammonium citrate deserves consideration. This salt is readily reduced to the ferrous state by light and is highly soluble at neutral pH. The citrate portion of the molecule is not oxidized by S. discophorus according to our manometric experiments. It may be that the iron of the complex is metabolically active or that the citrate functions as a chelating agent. Evidence is not yet available to permit a decision between these two possibilities.

It is not impossible that S. discophorus has a mixed autrtrophic-heterotrophic metabolism. The oxidation of iron and manganese may provide metabolic energy whereas the organic matter required for growth may be needed to supply organic carbon for cell synthesis. Such a mixed type of metabolism has been shown recently to occur in Desulfcvibrio desulfuricans (Mechalas and Rittenberg, 1960). This organism can derive energy for cellular synthesis either from hydrogen or nonassimilable isobutanol, whereas the cell carbon is provided by organic molecules in yeast extract which are unable to serve as energy sources.

IV. SUMMARY

In the present investigation, 11 pure cultures of Sphaerotilus discophorus were isolated from streams, rivers, and lakes. The hay infusion enrichment method involving the use of extracted hay, freshly precipitated $Fe(OH)_3$, $MnCO_3$ was employed successfully for the isolation of this organism.

Pure cultures were obtained by streaking the washed filaments on 0.5% peptone, 0.2% glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.005% $MnSO_4 \cdot H_2O$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.05% ferric ammonium citrate, 0.2 µg/ml thiamin, 0.02 ug/ml biotin and distilled water (pH 7.1). The colonies that develop have a characteristic dark brown coloration with filamentous, hairlike, curled edges. Young colonies are 2 to 5 millimeters in diameter. The dissociation of the cultures into glistening, hemispherical colonies with smooth edges and little or no pigmentation was observed on relatively rich media such as nutrient and yeast extract agar.

The individual filaments which compose the colonies are 10 to 15 µ wide and most of this width is due to inorganic deposits of iron and manganese oxides. The rod-shaped cells lie in a chain in the center of the filament encased in a tightly fitting, colorless, transparent, delicate primary sheath. The individual rods are 0.5 to 0.9 µ wide and 2 to 6 µ long. They are actively motile by means of a single polar flagellum.

S. discophorus can utilize a variety of carbon compounds, such as glucose, fructose, galactose, mannitol, sorbitol, sucrose, maltose, glycerol, and ethanol, as sources of carbon and energy. Slight growth occurred with xylose, arabinose, erythritol, lactate, and acetate. Little or no growth occurred in relatively rich media such as nutrient, yeast extract, Trypticase soy, and brain heart infusion broth or on corresponding agar media.

Organic nitrogen is essential for the growth of S. discophorus and can not be replaced by inorganic ammonium or nitrate salts.

By the use of vitamin-free casein hydrolysate in place of peptone, it has been shown that all of the strains require both thiamin and biotin for growth.

S. discophorus will not grow anaerobically and is a strict aerobe. The temperature range for the growth is 10 to 35 C with an optimum at 25 to 30 C. Growth will take place at 5 C but only after a prolonged incubation of about a month. The pH range for growth is 6.0 to 8.6 with an optimum between pH 7 to 8. S. discophorus is incapable of hydrolyzing dextrin, starch, cellulose, and gelatin. It decomposes hydrogen peroxide. Iron is essential for growth but its role in the metabolism of S. discophorus has not been established. Manganese is not essential for the growth in the standard medium although it is essential for the development of the typical dark brown color. Analyses showed that S. discophorus contains about 4 to 7% iron and about 1% manganese on a dry weight basis.

S. discophorus, like S. natans, accumulates a considerable amount of poly-β-hydroxybutyric acid in the cells. The polymer may account for as much as 40% of the dry weight of the cells.

S. discophorus, in resting cell suspensions, oxidizes a number of carbohydrates, members of the TCA cycle, and amino acids. In all cases, the extent of oxidation was only about 10% of theoretical for complete oxidation. t may be concluded that the organism is able to oxidatively assimilate these substrates to an unusually large extent.

PART II

ISOLATION AND IDENTIFICATION OF THE SUDANOPHILIC GRANULES OF SPHAEROTILUS NATANS

(see attached reprint)

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ISOLATION AND IDENTIFICATION OF THE SUDANOPHILIC GRANULES OF *SPHAEROTILUS NATANS*

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ABSTRACT

ROUF, M. A. (Washington State University, Pullman) AND J. L. STOKES. Isolation and identification of the sudanophilic granules of *Sphaerotilus natans*. J. Bacteriol. **83**:343-347. 1962.—The characteristic and prominent sudanophilic granules in five strains of *Sphaerotilus natans* were shown to be composed of poly-β-hydroxybutyric acid by the method of isolation, melting point determinations, and infrared absorption spectrum analyses. The polymer may account for 11.0 to 22.5% of the dry weight of the cells. It accumulates during active growth of *S. natans* and decreases sharply in amount after maximal growth. It accumulates to an equal extent in cultures with and without sheaths.

The cells of the filamentous, sheathed bacterium, *Sphaerotilus natans*, usually contain prominent and numerous refractile granules of variable size and shape. Büsgen (1894) concluded that the granules are composed of fatty materials since they are soluble in ether. By means of stains, also, Linde (1913) indicated that the granules contain lipids. In addition, he found volutin but not glycogen in the cells. According to Zikes (1915) and other investigators, young filaments are not granulated, but sudanophilic inclusion bodies appear in the cells as the filaments age. Zikes could not detect glycogen and, in contrast to Linde, he found little or no volutin. More recently, Stokes (1954) and Höhnl (1955) reported the common and abundant occurrence of sudanophilic granules in many strains of *S. natans*. According to Pringsheim (1949), the granules are of diagnostic value for this species.

S. natans will also deposit intracellular globules of sulfur when exposed to H_2S (Skerman, Dementjeva, and Carey, 1957).

It is now known that the sudanophilic granules of many genera and species of bacteria are composed mainly of poly-β-hydroxybutyric acid in association with a small amount of lipid material (Lemoigne, Delaporte, and Croson, 1944; Williamson and Wilkinson, 1958; Haynes et al., 1958; Forsyth, Hayward, and Roberts, 1958). It was of interest, therefore, to determine whether the sudanophilic granules of *S. natans* are also composed of poly-β-hydroxybutyric acid.

MATERIALS AND METHODS

Five strains of *S. natans* were used. Strains 13338, 13339, and B1493 are part of the group of nine strains previously investigated (Stokes, 1954). Strain 11021, isolated by Pringsheim, was obtained from the American Type Culture Collection. Strain P is one which we recently isolated from river water.

The cultures were grown at 25 C in shallow, stationary layers of broth. The medium contained 0.2% each of peptone and glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.01 M phosphate buffer (pH 7.1), and distilled water. The phosphate buffer was sterilized separately. The medium was distributed in 200-ml amounts into 2-liter Erlenmeyer flasks. One liter of each culture was grown for analysis.

After growth, the cells and filaments were harvested by centrifugation, washed twice with distilled water, and suspended in a known volume of water ranging from 60 to 100 ml; 2-ml samples were removed for the determination of the dry weight of the cells.

To isolate the sudanophilic granules, the cells from the remainder of the above aqueous suspension were collected by centrifugation, resuspended in 100 ml of 2 N H_2SO_4 and incubated at 100 C for 1 to 2 hr to hydrolyze and remove water-insoluble polysaccharides. The residue was collected, washed twice with distilled water in the centrifuge, and digested with the alkaline hypochlorite reagent of Williamson and Wilkinson (1958) for 12 to 18 hr at 35 C. This reagent digests and dissolves virtually all cellular com-

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ponents except poly-β-hydroxybutyric acid. The residue from the hypochlorite treatment was collected and washed three times in the centrifuge. To remove lipids, the residue was placed in about 100 ml of a mixture of one part of acetone and two parts of ether for several hours; this was followed by extraction with 30 ml of ether alone for several hours. The ether-extracted residue was treated with 30 ml of hot $CHCl_3$ to dissolve poly-β-hydroxybutyric acid, and the suspension was then centrifuged to remove $CHCl_3$-insoluble material. Finally, the supernatant fluid was dried to constant weight at 65 C. This procedure is essentially that used by Stamer et al. (1953) to isolate poly-β-hydroxybutyric acid from *Rhodospirillum rubrum*.

The infrared spectra of the isolated substances were determined with a Beckman IR-5 spectro-

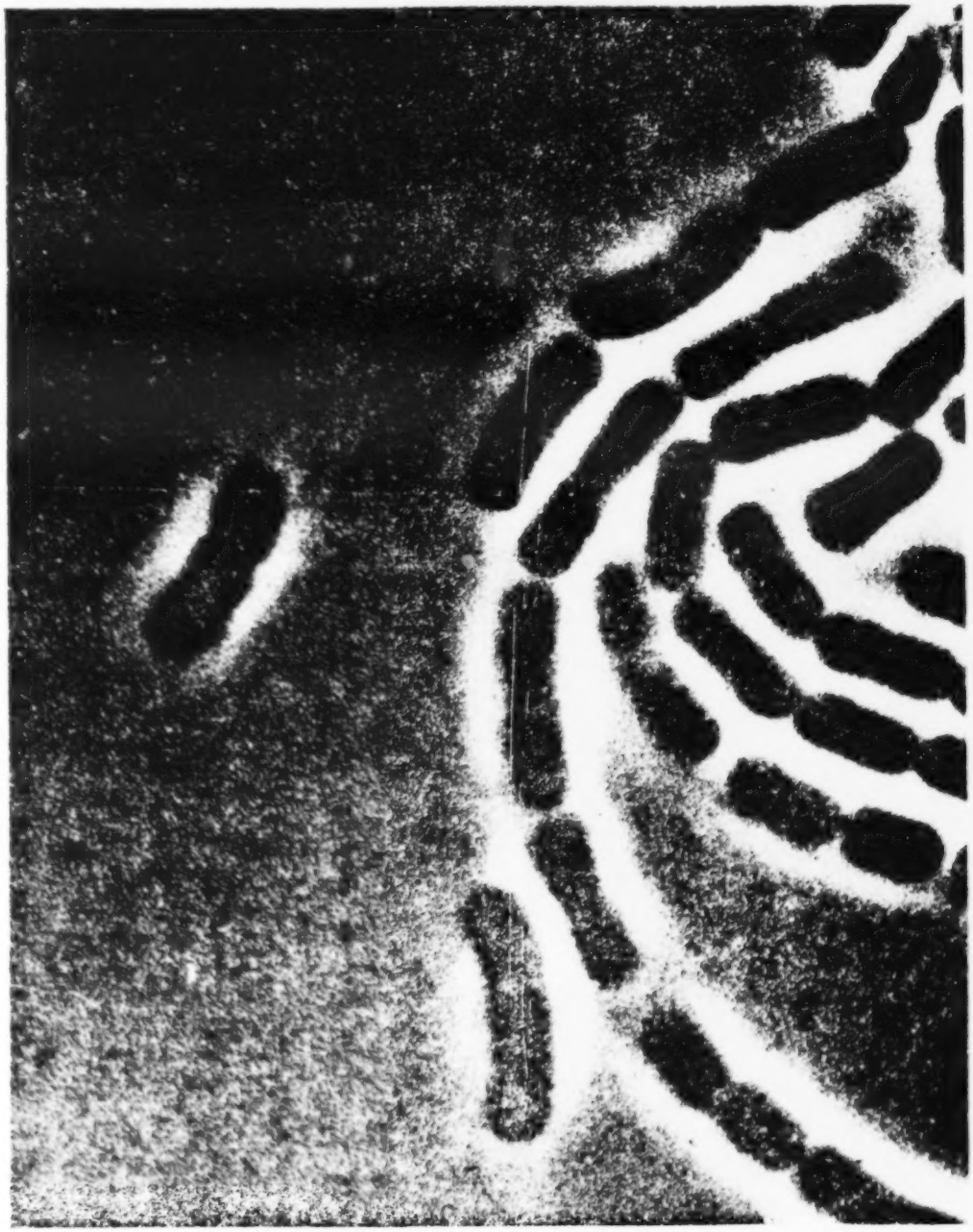

FIG. 1. *Sudanophilic granules in Sphaerotilus natans; living, unstained material photographed with the phase microscope (dark contrast). × 5,500.*

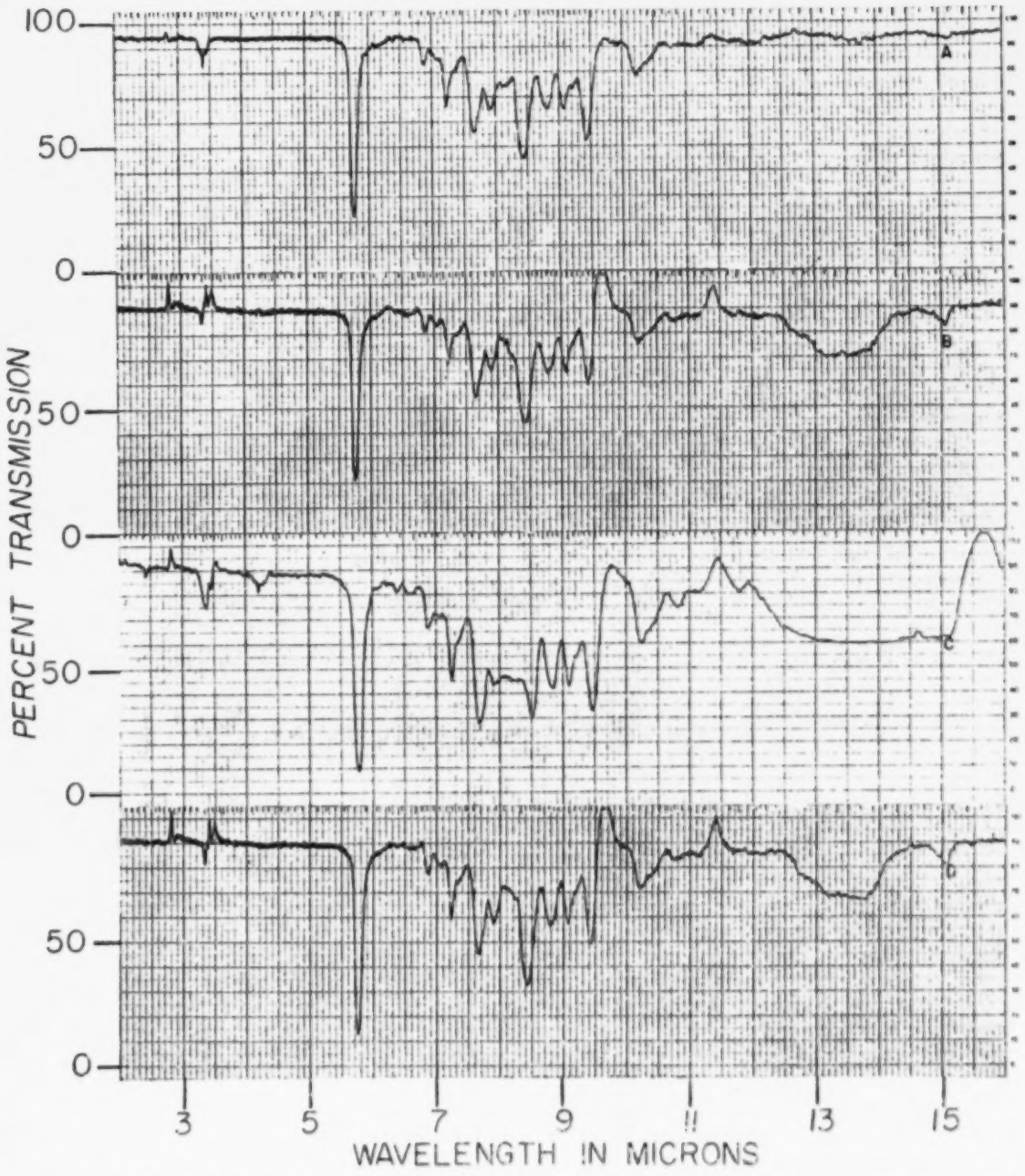

FIG. 2. *Infrared spectra of poly-β-hydroxybutyric acid isolated from Bacillus megaterium (A) and Sphaerotilus natans strains 11021 (B), 15338 (C), and B1493 (D).*

photometer. The $CHCl_3$ solutions used contained approximately 2.5 mg of material per ml.

RESULTS

All of the strains grew abundantly in the peptone-glucose-mineral salts medium, mainly as white, gelatinous surface pellicles. Maximal growth was reached usually within 2 to 3 days. Microscopically, the cultures consisted of characteristic *Sphaerotilus* filaments, i.e., long chains of rod-shaped cells enclosed in transparent, tightly-fitting tubes or sheaths. It was not possible, however, to be certain that the cells of strain 11021 were encased in a sheath. The individual cells of mature cultures of all of the strains contained, typically, numerous refractile gran-

ules. These appeared as dark inclusion bodies in the living, unstained cells when viewed with the phase microscope under dark contrast (Fig. 1). Also, they stained readily with Sudan Black in wet mounts. The granules may be small and well separated or may coalesce to form what appears to be a single, large, somewhat irregularly shaped body which may occupy one-half or more of the volume of the cell.

Direct microscopic observations of the staining of the granules in wet mounts with Sudan Black indicated that at least 90 to 95% of the granules were sudanophilic. Tests for volutin by Albert's staining method, as modified by Laybourne (1924), were inconclusive. Volutin granules, if present as reported by Linde (1913), can account for only a very small fraction of the total inclusion bodies in *Sphaerotilus*.

TABLE 1. *Amounts of poly-β-hydroxybutyric acid in strains of Sphaerotilus natans*

Strain no.	Incubation time*	Polymer content of cells
	hr	*mg per 100 mg dry wt*
13338	70	11.0
13339	69	12.6
P	60	15.8
B1493	68	18.7
11021	72	22.5

* At 25 C, except for 30 C for strain P.

In a series of experiments, each of the five strains of *Sphaerotilus* was grown in the peptone-glucose-mineral salts broth for approximately 3 days to obtain maximal growth. The cells were then harvested and analyzed for poly-β-hydroxybutyric acid by the procedure previously outlined.

We were able to isolate, from the final chloroform extract of every strain, a white, somewhat translucent, membranous material which exhibited the reported properties of poly-β-hydroxybutyric acid. It is resistant to alkaline hypochlorite. It melts in the range of 162 to 170 C (Williamson and Wilkinson, 1958). It has an infrared absorption spectrum similar to that of poly-β-hydroxybutyric acid isolated from *Bacillus megaterium* (Haynes et al., 1958). Figure 2 contains the spectra of the polybutyrate isolated from three of the strains of *S. natans* and, for comparison, that from *B. megaterium*, an organism known to form and accumulate the polymer. All four spectra are quite similar and exhibit a very prominent band at 5.7 μ, which is characteristic of poly-β-hydroxybutyric acid (Haynes et al., 1958).

The amounts of polybutyrate isolated from *S. natans* are relatively large and range from

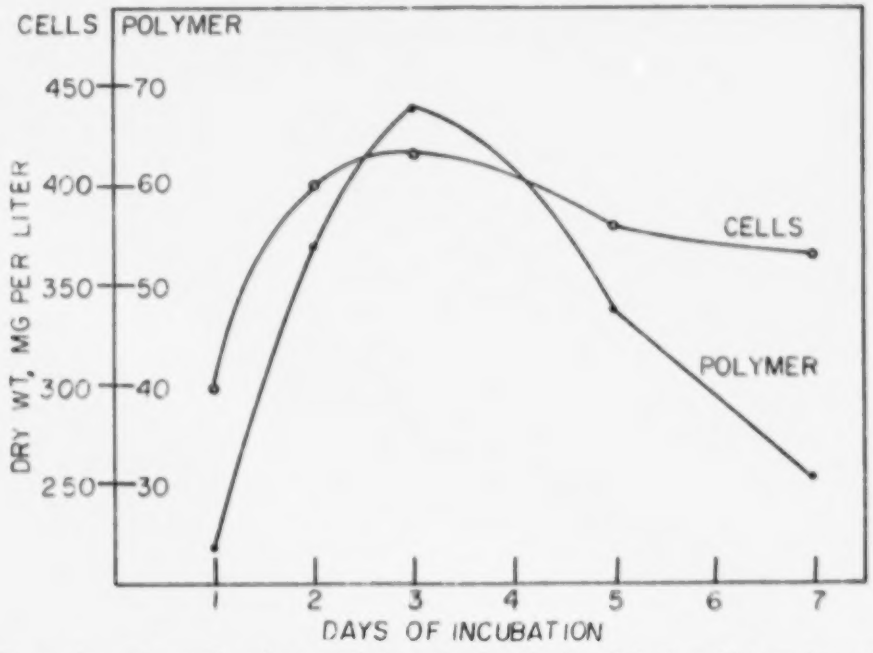

FIG. 3. *Comparison of growth and accumulation of poly-β-hydroxybutyric acid in Sphaerotilus natans B1493.*

11.0% of the dry weight of the cells of strain 13338 to 22.5% for strain 11021 (Table 1). These quantities are comparable to those from other high-yielding bacteria. Thus, *B. cereus* may contain from 10 to 43% polybutyrate (Williamson and Wilkinson, 1958); *R. rubrum*, 23% (Stanier et al., 1959); and *Pseudomonas methanica*, 29% (Kallio and Harrington, 1960).

The filamentous, sheathed form of *S. natans* can dissociate into one which does not produce a sheath (Pringsheim, 1949; Stokes, 1954). We found that substitution of 0.2% Bacto-Casamino acids for Bacto-peptone in the basal medium gave rise to cultures which did not form sheaths. Such cultures, however, contained as much polybutyrate as the typical sheathed cultures.

Effect of age. The amount of poly-β-hydroxybutyric acid in *S. natans* varies with the age of the culture. Growth and polybutyrate accumulation of strain B1493 as a function of days of incubation are plotted in Fig. 3. As growth proceeds and approaches the maximal value of 430 mg of dry weight of cells per liter of culture in 3 days, there is a concomitant increase in polymer from 23.6 mg per liter after 1 day to 56.4 mg after 2 days and 67 mg after 3 days. These values correspond, respectively, to 8.1, 14.1, and 15.6% of the polymer. On subsequent incubation for 5 days and 7 days, cell weights decrease only slightly, but there is a sharp drop in the polymer content to 47.2 mg (12.3%) in 5 days, and 30.5 mg (8.1%) in 7 days. Similar results were also obtained with strain 11021.

It is evident, therefore, that the amount of polymer in the cells reaches a maximum at the end of the exponential phase of growth, and then declines rapidly. These results support the current concept that poly-β-hydroxybutyric acid is a major, reserve-food product of bacterial cells. During early stages of growth, it accumulates as sudanophilic intracellular granules, and, later, is consumed by the cells as a source of carbon and energy (Macrae and Wilkinson, 1958*a*,*b*; Stanier et al., 1959).

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(2) List of Publications

1. Isolation and identification of the sudanophilic granules of Sphaerotilus natans.

   M. A. Rouf and J. L. Stokes
   J. Bacteriol. 83: 343-347, 1962.

2. Isolation and properties of Sphaerotilus discophorus.

   M. A. Rouf and J. L. Stokes
   Bacteriological Proceedings, p. 41, 1962.

3. Culture and physiology of Sphaerotilus discophorus and Sphaerotilus natans.

   M. A. Rouf
   Ph.D. Thesis, Washington State University, 1963.

(3) Staffing

J. L. Stokes, Professor, June 1, 1961--to present, approx. 20%.

M. A. Rouf, Graduate Student, June 1, 1961--June 1, 1963, 1/2 time

A. Johnson, Graduate Student, June 15, 1963--to present time, 1/2 time

Mrs. M. J. Powers, Laboratory Technician, January 2, 1963--to present time, 3/4 time

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1942-1943, Santa Barbara Hosp., Santa Barbara, Calif., Lab. Tech.

Note: Much of the information in the detailed report has not yet been published. It has been our intention to delay publication until relatively complete data are available on the various phases of the morphology, nutrition and physiology of Sphaerotilus, especially of S. discophorus. The morphological and growth aspects are now essentially complete and will be submitted for publication in the near future.